Journal of Surgical Oncology 1999;72:18–23

Identification of Semaphorin E Gene Expression in Metastatic Human Lung Adenocarcinoma Cells by mRNA Differential Display

MIREIA MARTÍN-SATUÉ[1] AND JERÓNIMO BLANCO[1,2]*
[1]Departament de Biologia Cel.lular, Institut de Recerca Oncològica, Barcelona, Spain
[2]Consejo Superior de Investigaciones Científicas, Barcelona, Spain

Synopsis: Human lung adenocarcinoma cell lines HAL-8Luc and HAL-24Luc differ in their metastatic potential. HAL-8Luc cells metastasize to lungs when injected either intravenously or intramuscularly. in mice while HAL-24Luc cells do not. The differential display method is used to identify genes differentially expressed between the two cell lines and the findings are extensively discussed.

Background: Lung cancer is the leading form of cancer in most countries, and metastasis is the main cause of death in oncological patients. The metastatic phenotype of tumor cells is the result of genetic events altering the RNA and protein expression of normal cells. Our objective was to identify genes expressed differentially between metastatic and nonmetastatic human lung adenocarcinoma cells that might be used as a prognostic factor.

Methods: The differential display technique was used to compare the RNA expression patterns distinguishing metastatic (HAL-8Luc) and nonmetastatic (HAL-24Luc) human lung adenocarcinoma cells, two genetically close cell lines.

Results: Differential expression of three cDNAs was confirmed by Northern blot analysis. Two sequences corresponding to a putative splicing factor and a proliferation-related factor cDNAs were underexpressed in the metastatic cells relative to the nonmetastatic ones. Interestingly, we found that human semaphorin E mRNA was several fold overexpressed in the metastatic cells. This recently identified gene encodes a protein whose expression has been related to several cell survival mechanisms as well as to immunosuppression.

Conclusion: Our results point to the relevance of semaphorin E in metastatic spread of human lung adenocarcinoma cells.

J. Surg. Oncol. 1999;72:18–23.

KEY WORDS: differential display PCR; HAL-24Luc cells; HAL-8Luc cells; nuclear RNA-binding protein; 6.6.5 mRNA sequence; MS3 mRNA sequence; H-sema E; semaphorin E

INTRODUCTION

Lung cancer is the leading cause of cancer death in most countries. In several areas, the annual incidence rates among males exceed 100 per 100,000, and adenocarcinoma is a major form of this pathology [1]. Much of the malignant neoplasm's lethality is directly attributable

Grant sponsor: Fondo de Investigaciones Sanitarias; Grant number: SAF 97/0115; Grant sponsor: Fundación Areces and Servei Català de la Salut, Generalitat de Catalunya.

*Correspondence to: Jerónimo Blanco, Dept. Biologia Cellular, Institut de Recerca Oncològica, Hospital Duran i Reynals, Autovia de Castelldefels Km. 2,7, 08907 Hospitalet de Llobregat, Barcelona, Spain. E-mail: jblanco@csub.scs.es

Accepted 7 June 1999

to the tumor's ability to develop secondary growths at distal organs [2]. It is, thus, of great clinical importance to identify novel genes associated with the processes of invasion and metastasis that could be targets for therapeutical intervention.

The differential display method developed by Liang and colleagues [3,4] is ideally suited to compare altered gene expression between eukaryotic cells. This technique, based on the polymerase chain reaction (PCR) amplifications of expressed RNA sequences, has already been used succesfully to identify genes differentially expressed in metastatic cells of diverse origins such as mammary carcinoma [5], brain tumors [6], medullary thyroid carcinoma [7], and melanoma [8, 9].

HAL-8Luc and HAL-24Luc are human lung adenocarcinoma cell lines characterized by their different metastatic potentials [10]. When injected either intravenously or intramuscularly into in athymic mice, HAL-8Luc cells developed lung metastasis while HAL-24Luc cells did not metastasize. Both cell lines were derived from a common parental cell line [11] and, consequently, have a close genetic background; therefore they provide a convenient system to study changes in gene expression that could be related to their metastatic behaviour. We have previously described that enhanced expression of α(1,3)-fucosyltransferase genes in the metastatic HAL-8Luc cells, compared with HAL-24Luc cells, contributes to the adhesive capacity of these cells to the activated endothelium and correlates with their lung colonization potential [10,12]. We show here for the first time the use of the differential display method to identify genes differentially expressed in these two cell lines that could be related to human lung adenocarcinoma cell invasiveness.

MATERIALS AND METHODS

Cell Culture

Human lung adenocarcinoma cell lines HAL-8Luc (metastatic) and HAL-24Luc (nonmetastatic) [10] were maintained in RPMI 1640 (Bio-Whittaker, Verviers, Belgium) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Bio-Whittaker), 2 mM L-glutamine (Bio-Whittaker), penicillin (100 U/ml), streptomycin (100 μg/ml) and 300 μg/ml geneticin (G418; Life Technologies, Grand Island, NY) in a humidified atmosphere of 5% CO_2 at 37°C.

mRNA Isolation and Primers

mRNAs were extracted from confluent HAL-8Luc and HAL-24Luc cell cultures by using the PolyATtract System 1000 kit from Promega (Madison, WI). Oligonucleotide primers were obtained from Pharmacia Biotech Europe (Barcelona, Spain). Downstream primers were $T_{11}AA$ and $T_{12}AG$, and 10-mer arbitrary primers were MM1, 5′- CATAAGCAGG-3′; MM2, 5′-TACGATGACG-3′; 1374, 5′-GTGGCCGAGG-3′.

Differential Display

Differential-display reverse-transcription technique (DDRT) was performed according to the method of Liang et al. [4]. mRNA (100–700 ng) was reverse-transcribed with 300 U of murine Maloney leukemia virus reverse transcriptase (Promega, Madison, WI) in the presence of 2.5 μM anchored downstream primer (either $T_{11}AA$ or $T_{12}AG$) in a 20-μl reaction. This reverse transcription product (2 μl) was PCR-amplified in the presence of [α-^{33}P]-dATP with the matching downstream primer and one of the 10-mer upstream primers (MM1, MM2, or 1374), using the following cycling parameters: 94°C for 30 sec, 40°C for 2 min, and 72°C for 30 sec, for 40 cycles, followed by 5 min at 72°C. A 3.5-μl aliquot of PCR product was mixed with 2 μl of loading dye and incubated for 2 min at 95°C before loading onto a 6% DNA sequencing gel. Electrophoresis was conducted for about 2.5 h at 55 W, constant power. The gels were dried and exposed to Hyperfilm MP (Amersham, Amersham, UK). Fragments of cDNA corresponding to bands that were differentially displayed between sets of cells were recovered from the gel by boiling the gel segment in dH_2O for 15 min. The eluted DNA was reamplified with the same set of primers and the same cycling parameters used to generate the original PCR product. The amplified fragments were cloned into the pCRII vector using the TA Cloning kit (Invitrogen, San Diego, CA), and sequenced using the T7 Sequenase 2.0 DNA sequencing Kit (Amersham). Sequences were compared to known nucleotide sequences in GenBank by use of the Basic Local Alignment Tool (BLAST) program.

Northern Blots

Three micrograms of poly(A)$^+$ RNA from HAL-8Luc and HAL-24Luc cells and 2 μg of RNA molecular weight markers (United States Biochemical, Cleveland, OH) were fractionated by electrophoresis in a 1.2% denaturing agarose gel containing 1.8% (v/v) formaldehyde and then transferred to nylon membranes (Hybond-N$^+$; Amersham). The membranes were hybridized with ^{32}P-labeled cDNA probes in a solution containing 50% (v/v) formamide, 5 × standard saline citrate (SSC), 50 mM sodium phosphate buffer, pH 6.5, 250 μg/ml sheared salmon sperm DNA, 10× Denhart's solution, and 10% dextran sulphate overnight at 42°C. Following hybridization, the membranes were washed twice for 10 min at room temperature in 2 × SSC, 0.1% sodium dodecyl sulfate (SDS), and once in 0.2 × SSC, 0.1% SDS for 1 h at 68°C. Finally, the membranes were autoradiographed by exposure to Hyperfilm MP (Amersham). Blots were stripped and reprobed with human β-actin cDNA as a control for RNA integrity and loading consistency. Band intensity was analyzed with the Molecular Analyst/PC 1.4 software (BioRad, Hercules, CA) and calibrated by comparison with the β-actin bands.

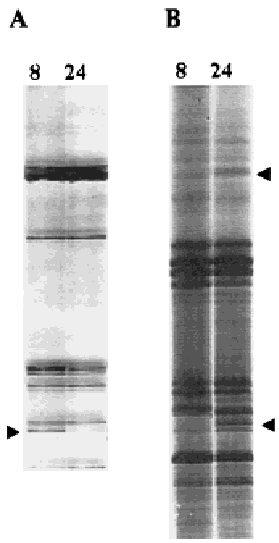

Fig. 1. mRNA differential display gel patterns from metastatic (8) and nonmetastatic (24) human lung adenocarcinoma cells using two different primer combinations: T_{11}AA-1374 (**A**) and T_{12}AG-MM1 (**B**). Differentially expressed cDNA fragments are indicated by arrowheads.

RESULTS

To identify genes showing altered expression in the metastatic human lung adenocarcinoma HAL-8Luc cells, we compared mRNA expression by differential display using poly(A)$^+$ RNA extracted from HAL-8Luc cells and their nonmetastatic counterpart HAL-24Luc cells. Three 10-mer arbitrary primers were used in combination with either T_{11}AA or T_{12}AG downstream primers. Figure 1 shows typical DDRT-PCR amplifications obtained using two separate primer combinations. Differentially expressed PCR products are indicated. Once reamplified and cloned, the PCR-identified products were used as probes in Northern blot analysis for confirmation of differential expression and were full-length sequenced (Table I). We analyze in this work three such PCR products: MS3, a 274-bp sequence overexpressed in the metastatic cells, and the 6.6.5 (342 bp) and 18.1.5 (330 bp) sequences, both underexpressed in the same cells. cDNAs corresponding to these sequences were isolated from the gel, reamplified, and cloned.

Northern Blot Analysis

To further establish by an independent procedure that the candidate sequences were differentially expressed, mRNA from metastatic and nonmetastatic cells was analyzed by Northern blot. Probes were the cDNA clones derived from the differential display analysis (Fig. 2).

Significantly different expression levels for the three sequences were confirmed. Probe 6.6.5 identified two transcripts, 4 and 1.9 kb, whose expression was reduced in HAL-8Luc cells (3- and 1.5-fold, respectively). Probe 18.1.5 hybridized with a 2.1-kb message whose expression appeared also diminished (1.5-fold) in those cells. Finally, the MS3 sequence that was 7-fold overexpressed in the metastatic compared with the nonmetastatic cells recognized a 5.2-kb message.

DNA Sequence Analysis

Differentially expressed cDNAs were full-length sequenced and compared to GenBank sequences (Table I). Sequence MS3 was identical to human semaphorin E mRNA (GenBank accession no. AB000220), sequence 18.1.5 was 98.5% homologous to a 54-KDa protein mRNA (GenBank accession no. U02493), and the 6.6.5 sequence showed a 93.3% homology with a proliferating cell-associated mRNA from *Rattus norvegicus* intestinal epithelium (GenBank accession no. U21718).

DISCUSSION

Cancer metastasis requires a long series of sequentially interrelated steps, the failure or insufficiency of any one of which may abort the process. In a broad sense, besides the need to avoid the immune system, these steps include (1) progressive growth of neoplastic cells after the initial transforming event, (2) establishment of a tumor neocapillary network from the surrounding host tissue, (3) entry to the circulation, (4) adhesion of tumor cells that survive the circulation to the capillary beds of organs, (5) extravasation, and (6) proliferation within the organ parenchyma [13]. Each of these steps is in itself the result of a complex series of events that result from the alteration of normal gene expression. The differential display method is ideally suited for the detection of changes in gene expression that may result in the metastatic phenotype of tumor cells.

We describe the identification by differential display of three genes differentially expressed between the metastatic human lung adenocarcinoma cell line HAL-8Luc and the nonmetastatic but genetically close HAL-24Luc cells. The 6.6.5 and 18.1.3 mRNA sequences were underexpressed in HAL-8Luc cells, while the MS3 mRNA sequence was overexpressed in the same cells.

Sequence 18.1.5 is 98.5% homologous to the human mRNA sequence encoding p54^{nrb} (for nuclear RNA-binding protein, 54 kDa). Although the function of this protein remains unknown, it contains two RNA recognition motifs and shares high homology with human splicing factor PSF. p54^{nrb} and PSF were identified in HeLa cells and probably function as a general and/or regulatory splicing factor [14]. As has been previously reported, alternative splicing of a single gene may lead to unbalanced expression of some mRNA isoforms related to neoplastic progression and metastatic promotion [15–17].

Sequence 6.6.5 shares high homology with a proliferating cell-associated mRNA from rat intestinal epithe-

TABLE I. Summary of Clones Differentially Expressed Between Metastatic and Nonmetastatic Cell Lines

Clone	Relative expression in metastatic cells	Northern blot confirmation	Identity; homology
18.1.5	Reduced	Confirmed	Human 54-kDa protein mRNA (U02493); 325/330, 98.5%
16.1.3	Reduced	Not confirmed	Human mRNA for β-actin (X00351); 240/240, 100%
18.3.1	Reduced	Confirmed	Human mRNA for fibrillarin (M59849); 283/294, 96.2%
16.1.4	Reduced	Not confirmed	Not determined
17.4.1	Augmented	Not confirmed	Not determined
17.2.1	Reduced	Not detectable	Unknown
17.3.1	Augmented	Not confirmed	Not determined
6.6.5	Reduced	Confirmed	*Rattus norvegicus* clone c426 intestinal epithelium cell-associated mRNA (U21718); 319/342, 93.3%
136.1.4	Augmented	Not detectable	Unknown
136.3.4	Augmented	Not confirmed	Not determined
MS3	Augmented	Confirmed	Human semaphorin E mRNA (AB000200); 274/274, 100%
MS1.8	Augmented	Not detectable	Unknown
MS2.4	Augmented	Not confirmed	*Homo sapiens* chromosome 17, clone HCIT542B22 (AC004253); 156/166, 93%
MS4.6	Augmented	Not confirmed	*Homo sapiens* mitochondrial genoma (V00662); 166/174, 95%
MS7.2	Reduced	Not confirmed	Bovine gamma globin gene (M63452); 171/184, 92%

lium [18]. To date, the corresponding protein has yet to be identified. However, the high homology shared by the human 6.6.5 sequence and its rat homologue suggests the existence of a new, highly conserved family of proliferating factors in epithelial cells. It is conceivable that altered expression of the activating and/or inhibiting factors implicated in the highly regulated process of cell proliferation would contribute to tumor metastasis. In this context, it has been shown that decreased expression of the human KAI-1 metastasis-suppressor gene resulted in the progression of human prostate and possibly lung and breast cancers as well [19].

Sequence MS3 was identical to the recently identified human semaphorin E (H-sema E) mRNA. The semaphorin gene family codes for transmembrane and secreted proteins ~750 aa long characterized by a ~500 aa extracellular domain termed the semaphorin (sema) domain, which is conserved between invertebrates and vertebrates [20]. The best-characterized function of this protein family is the repulsive guidance of nerve axons during development [21–25] but its function in nonneural systems is largely unknown. There are indications that mouse semaphorin D and E are involved in the histogenesis of tissues other than neural [23], and several reports indicate that semaphorins also participate in diverse cell survival mechanisms [26, 27]. Homologues to the sema domain of semaphorins have been found in two poxvirus-encoded proteins involved in the immune suppression of the host [28], which has lead to the speculation that semaphorins may function as natural suppressants of the immune system [22].

H-sema E is a secreted protein. Transfection of semaphorin E conferred drug-resistant phenotype to cisplatin-sensitive human ovarian cancer cells, probably by acting as an autocrine factor bound to undetermined cell surface receptor(s) [27]. In addition, the same authors described aberrant overexpression of H-sema E in 33.3% of recurrent squamous cell carcinomas, including those of pulmonary origin, removed at autopsy after extensive radiochemotherapy. These results suggest the participation of H-sema E in the carcinogenesis and progression of certain human tumors. Increased expression of H-sema E has also been related to the pathogenesis of rheumatoid arthritis, where it has been suggested this protein has a role as regulator of inflamatory processes [29,30].

On the basis of these previous findings, we suggest that in human lung adenocarcinoma cells, sema E may be involved in tumor cell survival mechanisms, participating in the immunological regulation of tumor spread by helping to avoid or subvert immune surveillance. This is further reinforced by our observation of a noticeable absence of lymphocytes in intramuscularly generated HAL-8Luc tumors, in contrast with the high lymphocyte infiltration observed in the HAL-24Luc derived primary tumors [10]. These results support a role of sema E in the metastatic phenotype of tumor cells as an immunological regulator of tumor progression.

CONCLUSIONS

In summary, our results demonstrate that mRNA differential display is a valuable tool for identification of potential metastasis-related genes in this human lung adenocarcinoma system. Our results also reinforced recent studies pointing to the usefulness of sema E as a prognostic factor in human lung adenocarcinomas.

ACKNOWLEDGMENTS

We thank Dr. O. Matsuo (Kinki University School of Medicine, Osakasayama City, Japan) for kindly providing HAL-8 and HAL-24 cell lines, Dr. J. García-Valero (Departament Biologia Cellular, Facultat de Biologia, Universitat de Barcelona, Barcelona, Spain) for valuable

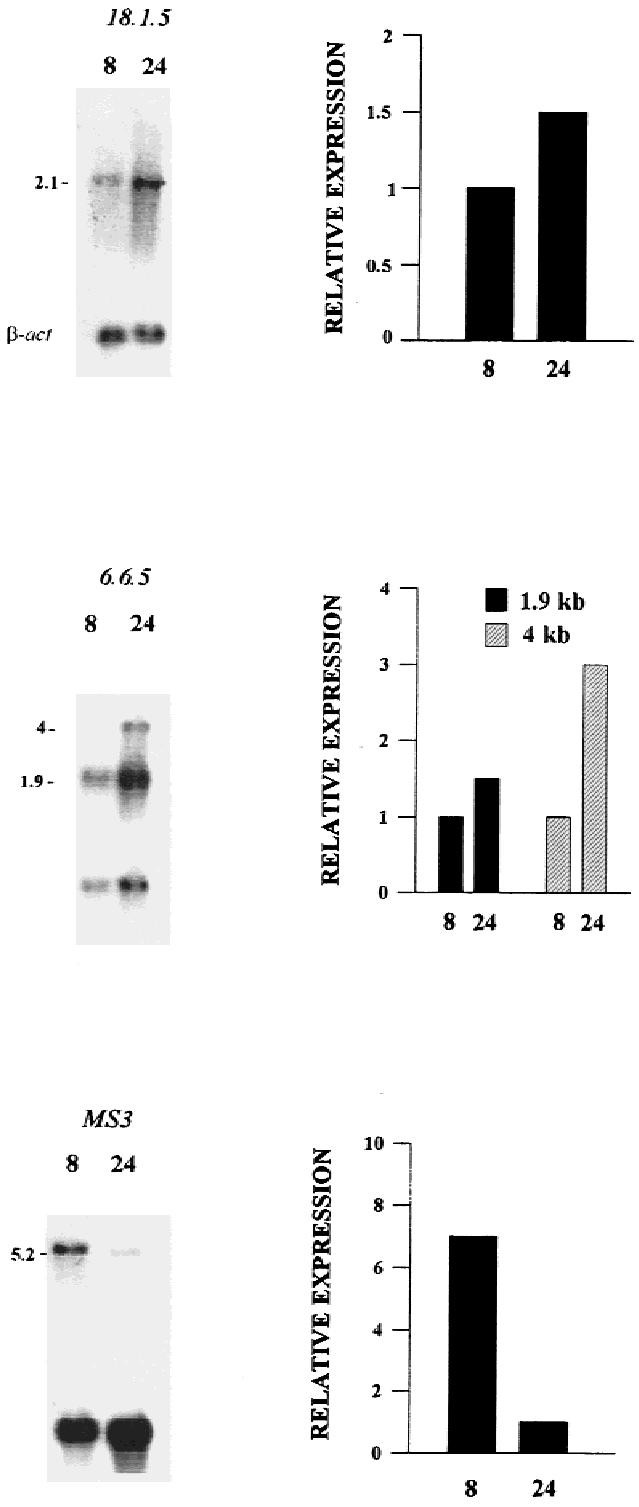

Fig. 2. Northern blot analysis of metastatic (8) and nonmetastatic (24) human lung adenocarcinoma cell mRNA using the 18.1.5, 6.6.5, and MS3 sequences as probes. Poly(A)$^+$ RNA (3 μg) was electrophoresed, blotted, and hybridized with the ^{32}P-labeled 18.1.5, 6.6.5, and MS3 cDNA probes as described in Materials and Methods. After a first autoradiographic exposure, the probes were stripped out, and the blots were rehybridized with the β-actin probe (β-act). Band sizes are indicated in kilobases at the left of each set. The bar graphs represent the relative expression level of each mRNA species in the respective cell lines. Relative mRNA levels were calculated after correction of gel loading differences, using the β-actin gene expression as standard.

critical review of the manuscript, and Rosabel Marrugat (Institut de Recerca Oncològica, Barcelona, Spain) for excellent technical assistance.

REFERENCES

1. Blot WJ, Fraumeni JF Jr: Cancers of the lung and pleura. In Schottenfeld D, Fraumeni JF Jr (eds): "Cancer Epidemiology and Prevention." New York: Oxford University Press, 1996:637–665.
2. Woodhouse EC, Chuaqui RF, Liotta LA: General mechanisms of metastasis. Cancer 1997;80:1529–1537.
3. Liang P, Pardee AB: Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. Science 1992; 257:967–971.
4. Liang P, Averboukh L, Pardee AB: Distribution and cloning of eukaryotic mRNAs by means of differential display: refinements and optimization. Nucleic Acids Res 1993;21:3269–3275.
5. Liu Z, Brattain MG, Appert H: Differential display of reticulocalbin in the highly invasive cell line, MDA-MB-435, versus the poorly invasive cell line, MCF-7. Biochem Biophys Res Commun 1997;231:283–289.
6. Sehgal A, Keener C, Boynton AL, et al.: Characterization of C4-2 as a tumor-suppressor gene in human brain tumors. J Surg Oncol 1997;64:102–108.
7. Musholt TJ, Goodfellow PJ, Scheumann FW, et al.: Differential display in primary and metastatic medullary thyroid carcinoma J Surg Res 1997;69:94–100.
8. Lee JH, Welch DR: Identification of highly expressed genes in metastasis-suppressed chromosome 6/human malignant melanoma hybrid cells using subtractive hybridization and differential display. Int J Cancer 1997;71:1035–1044.
9. Duncan LM, Deeds J, Hunter J, et al.: Down-regulation of the novel gene melastatin correlates with potential for melanoma metastasis. Cancer Res 1998;58:1515–1520.
10. Martín-Satué M, Marrugat R, Cancelas JA, Blanco J: Enhanced expression of alpha(1,3)-fucosyltransferase genes correlates with E-selectin-mediated adhesion and metastatic potential of human lung adenocarcinoma cells. Cancer Res 1998;58:1544–1550.
11. Inufusa H, Kojima N, Yasutomi M, Hakomori S: Human lung adenocarcinoma cell lines with different lung colonization potential (LCP), and a correlation between expression of sialosyl dimeric Le(x) (defined by MAb FH6) and LCP. Clin Exp Metastasis 1991;9:245–257.
12. Martín-Satué M, de Castellarnau C, Blanco J: Overexpression of α(1,3)- fucosyltransferase VII is sufficient for the acquisition of lung colonization phenotype in human lung adenocarcinoma HAL-24Luc cells. Br J Cancer 1999;80(8):1169–1174.
13. Fidler IJ: Critical factors in the biology of human cancer metastasis: twenty-eighth G.H.A. Clowes memorial award lecture. Cancer Res 1990;50:6130–6138.
14. Dong B, Horowitz DS, Kobayashi R, Krainer AR: Purification and cDNA cloning of HeLa cell p54nrb, a nuclear protein with two RNA recognition motifs and extensive homology to human splicing factor PSF and Drosophila NONA/BJ6. Nucleic Acids Res 1993;21:4085–4092.
15. Harwood CA, Green MA, Cook MG: CD44 expression in melanocytic lesions: a marker of malignant progression?. Br J Dermatol 1996;135:876–882.
16. De Rossi G, Marroni P, Paganuzzi M, et al.: Increased serum levels of soluble CD44 standard, but not of variant isoforms v5 and v6, in B cell chronic lymphocytic leukemia. Leukemia 1997; 11:134–141.
17. Siebert PD, Huang BC: Identification of an alternative form of human lactoferrin mRNA that is expressed differentially in normal tissues and tumor-derived cell lines. Proc Natl Acad Sci USA 1997;94:2198–2203.
18. Sykes DE, Weiser MM: The identification of genes specifically expressed in epithelial cells of the rat intestinal crypts. Differentiation 1992;50:41–46.
19. Dong JT, Isaacs WB, Barrett JC Isaacs JT: Genomic organization of the human KAI1 metastasis-suppressor gene. Genomics 1997; 41:25–32.
20. Kolodkin AL: Semaphorins: mediators of repulsive growth cone guidance. Trends Cell Biol 1996;6:15–22.

21. Kolodkin AL, Matthes DJ, O'Connor TP, et al.: Fasciclin IV: sequence, expression, and function during growth cone guidance in the grasshopper embryo. Neuron 1992;9:831–845.
22. Luo Y, Raible D, Raper JA: Collapsin: a protein in brain that induces the collapse and paralysis of neuronal growth cones. Cell 1993;75:217–227.
23. Püschel AW, Adams RH, Betz H: Murine semaphorin D/collapsin is a member of a diverse gene family and creates domains inhibitory for axonal extension. Neuron 1995;14:941–948.
24. Matthes DJ, Sink H, Kolodkin AL, Goodman CS: Semaphorin II can function as a selective inhibitor of specific synaptic arborizations. Cell 1995;81:631–639.
25. Bagnard D, Lohrum M, Uziel D et al.: Semaphorins act as attractive and repulsive guidance signals during the development of cortical projections. Development 1998;125:5043–5053.
26. Hall KT, Boumsell L, Schultze JL, et al.: Human CD100, a novel leukocyte semaphorin that promotes B-cell aggregation and differentiation. Proc Natl Acad Sci USA 1996;93:11780–11785.
27. Yamada T, Endo R, Gotoh M, Hirohashi S: Identification of semaphorin E as a non-MDR drug resistance gene of human cancers. Proc Natl Acad Sci USA 1997;94:14713–14718.
28. Ray CA, Black RA, Kronheim SR, et al.: Viral inhibition of inflammation: cowpox virus encodes an inhibitor of the interleukin-1 beta converting enzyme. Cell 1992;69:597–604.
29. Mangasser-Stephan K, Dooley S, Welter C, et al.: Identification of human semaphorin E gene expression in rheumatoid synovial cells by mRNA differential display. Biochem Biophys Res Commun 1997;234:153–156.
30. Seki T, Selby J, Häupl T, Winchester R: Use of a differential subtraction method to identify genes that characterize the phenotype of cultured rheumatoid arthritis synoviocytes. Arthritis Rheum 1998;41:1356–1364.